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(21) International Application Number: PCT/SE96/00902 (22) International Filing Date: 3 July 1996 (03.07.96) (30) Priority Data: 9502409-7 1 November 1995 (01.11.95) SE (71)(72) Applicant and Inventor: ANDERSSON, Birger [SE/SE]; Jungfrugatan 45, S-114 44 Stockholm (SE). (74) Agents: BERG, S., A. et al.; H. Albiöns Patentbyrå AB, P.O. Box 3137, S-103 62 Stockholm (SE).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: A PROCESS FOR IN VITRO ANALYSIS OF TOXIC AND ALLERGENIC SUBSTANCES (57) Abstract <p>The invention concerns a process for in vitro evaluation of a potentially allergenic or tissue irritating substances, characterized in that blood cells are cultivated in the presence of serial dilutions of the substance whereby the highest concentration of the substance being non toxic to the cells is serially diluted, cell proliferation is measured and the presence of cytokines is measured whereby: the presence of one or more of the alarm cytokines of class 0 only is an indication of tissue damage and chemical toxic effects; the presence of one or more alarm cytokines of class 0 and possibly one or more cytokines of class IV type but not neopterin is an indication of delayed type hypersensitivity such as cellular immunity, delayed allergy and contact eczema; and the presence of one or more alarm cytokines of class 0 and at least neopterin and possibly one or more cytokines of class I is an indication of immediate type hypersensitivity such as asthma, hay fever, urticaria and rhinitis. The invention also concerns use of neopterin and IL-8 as analytic substances for distinguishing between the immune reactions type I and IV and a kit for analysis.</p>		

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A process for in vitro analysis of toxic and allergenic substances

5 The present invention concerns a process for in vitro analysis of toxic and allergenic substances. Substances intended for use as pharmaceuticals, food additives, cosmetic or hygienic products, industrial chemicals and other substances are analysed for adverse reactions. Such reactions may be allergic reactions, skin irritating effects and toxic effects.

10 Today such tests are performed in vivo on animals. Animals are expensive to raise and keep. Further, some tests may involve suffering of the animals.

15 The present invention provides a process for in vitro analysis of the adverse effects of substances. The analysis of the substances is performed on blood cells from warm blooded animals. No animals are involved since the substances are tested on human cells i.e. cells from the same species as they are intended for. Some substances may not have the same effect on different species, and tests performed on animals may give false results. Therefore the test is preferably performed with human blood.

20 Thus, the method will be offered as an alternative to animal tests for food additives, cosmetic or hygienic products, industrial chemical, drugs and other substances where adverse reactions are to be avoided.

25 In vitro evaluation of toxic effects on the immune system has been tested on splenocytes from mice ("In vitro evaluation of drug-induced toxic effects on the immune system as assessed by proliferative assays and cytokine production", M. Pallardy et. al. Eur. Cytokine Net., Vol. 2 No 3, May-June 1991, pp. 201-206). The method was poorly effective detecting molecules inducing autoimmunity and hypersensitivity.

It has now turned out that according to the present invention it is possible to analyse adverse tissue reactions to foreign substances in vitro by cultivating them with human whole blood, prevented from coagulating and analysing the cytokine produced by the human cells.

Before a substance has been in use and before the target group has been exposed the present method offers the possibility to predict whether a risk for adverse reactions of inflammatory or allergic type will occur.

The invention provides an interpretation key for evaluating the adverse tissue reactions to foreign substances.

Summary of the invention: The method is defined in claim 1 and is based on cultivation of blood cells in vitro. The substance to be evaluated is added to the blood culture. If the reactive cells of the blood recognises the test material as foreign or damaging a production of inflammatory signal substances, cytokines occurs. The blood contains different white blood cells with different functions in the response to foreign substances. The different types of inflammatory cells secrete different repertoires of cytokines. The patterns, (profiles) of the secreted cytokines indicate what compartment of the immune system that has been activated. Thereby is it also possible to predict what kind of adverse reaction to be expected from a substance if administered to humans or experimental animals. Some substances will induce a typical cytokine profile driving the immune system towards IgE mediated allergy, thus indicating a potential of those substances to evoke asthma, hay fever and urticaria. Other substances will induce a cytokine pattern driving the immune system to cellular immunity thus having the potential to result in contact excema as an adverse reaction. Finally, a chemical or toxic effect will result in a cytokine pattern typical of alarm reactions seen in tissue damage and operations. Thus, the concept that the present method is based on is to use an interpretation of the cytokine pattern that a

substance can induce in blood in vitro cells to predict whether the substance has the potential to cause allergy of different types or is generally hazardous to induce tissue damaging or toxic effects.

5

We classify adverse tissue reactions to foreign substances as follows.

10

Class 0: Tissue damage. Unspecific chemical, mechanical or physical damage.

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Class I: Allergic immune reaction type I. This is also named immediate type hypersensitivity and is mediated by IgE antibodies produced specifically to the foreign substance. An acute inflammatory reaction is produced, histamine is often produced and examples of symptoms are asthma hay fever, urticaria and rhinitis. This type requires that the substance can induce a fully mature immune response where all components of the immune system participates. This is also considered as a final step immune reaction.

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Class IV: Inflammatory immune reaction type IV. This is also named delayed type hypersensitivity and is mediated by sensitised T lymphocytes type TH-1, considered as a first step type immune reaction. Allergic contact dermatitis is an example of this type of reaction.

30

Class 0 is our own classification of unspecific damage to the immune system. For Class I and Class IV see "Immunology" by Ivan Roitt, Jonathan Brostoff and David Male, Gower Medical Publishing London - New York, 1989, page 19.1-19.20 and 22.1-22.10, which is incorporated as a reference.

35

Three main types of cytokine profiles have been identified in the present work.

Class 0 type: Secretion of alarm cytokines indicating damage to connective tissue, fibroblasts, endothelial cells,

epithelial cells and unspecific inflammatory white blood cells. Members of this group are IL-1, IL-6, IL-12 and TNF.

Class I type: Secretion of cytokines of an immune response type from lymphocytes and inflammatory cells. These includes cytokines from Class 0 and in addition IFN-gamma, Neopterin, IL-2 and IL-10. Theoretically as known from animal in vivo studies also IL-4 and IL-5 should be produced here, but these substances are notoriously difficult to determine and are therefore not routinely included in our test protocols.

Class IV type: Secretion of the cytokines of Class 0 unspecific type and in addition IL-2, IL-8, IL-10 and IFN-gamma.

A finding that could not predicted was that the complete final type immune response Class I drives to Neopterin production whereas the Class IV primary type immune response does not stimulate the immune system as long as to Neopterin production.

In the three groups of cytokines listed above only preferably selected representative members are listed. The groups may contain other cytokines to be used and the patent is claimed for the use of analysis of all substances listed in The Cytokine Facts Book, Eds Callard R.E. and Gearing, A.J.H., Academic Press 1994 hereby incorporated as a reference and future updated issues to predict the grade of maturity of the response that a substance has the potential to evoke. At present 50 substances.

Interleukins

IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15

Other Cytokines (in alphabetical order)

BDNF, CNTF, EGF, Epo, FGF, G-CSF, GM-CSF, I-309/TCA-3, γ IP-10, IFN α , IFN β , IFN γ , LIF, LT(TNF β), MCP-1, 2 and 3,

M-CSF, MIF, MIP-1 α , MIP-1 β , MIP-2, NGF, NT-3, NT-4, OSM, PBP, PBSF, PDGF, PF-4, RANTES, SCF, TGF α , TGF β , TNF α , Tpo, VEGF

According to the invention the presence of inflammatory
5 immune reaction type IV is preferably analysed by using IL-8.
It has turned out that IL-8 is developed and is present in
raised levels during type IV reactions.

10 Neopterin being present in higher amounts when type I reac-
tion is at hand, IL-8 and/or neopterin can especially be used
to distinguish between the inflammatory immune reaction type
IV and the allergic immune reaction type I.

15 Based on the outcome of the in vitro test, the cytokine
pattern induced by a the substance under investigation, a
prediction will be made regarding the potential of the sub-
stance to cause adverse reaction if humans or animals are
exposed to it. Whole blood, preferably venous blood is used.
The blood is prevented from coagulating. This can be done by
20 the use of endotoxin free heparin tubes. Other methods may
also be used such as addition of EDTA or citrate. Alter-
natively the blood can be shaken with glass beads.

25 The leukocyte counts in undiluted blood ranges between 3×10^9
and 7×10^9 per litre. Around 30% of these are lymphocytes and
5-10% are monocytes the rest being polymorfonuclear cells.
Total leukocyte numbers and differential count is determined
in a Coulter STKS cell haematology analyser before further
procedure.

30 Whole blood is diluted serially from 1:1-1:1000, preferably
1:2-1:100 in a culture medium, preferably a tissue culture
medium such as RPMI 1640 supplemented with glutamine. Nor-
mally 1,5-5mM, preferably 2mM is used. The blood is most
35 often diluted 1:5, 1:10 1:20 or continued to 1:40, 1:80,
1:100.

Antibiotics are preferably added to prevent the growing of unwanted microorganisms. Penicillin and streptomycin may be added in concentrations of 25-100U/ml, preferably 50U/ml.

5 Mitogens or substances with known effects on the immune system can be used as positive controls to give better results. Any mitogen as mentioned in Daniel P. Stites: Clinical Laboratory Methods for Detection of Antigens & Antibodies in Basic and Clinical Immunology, Lange Medical Publications, Los Altos California, 1984 may be used. This
10 reference is incorporated in this description by reference. Phytohemagglutinin (PHA-L) is preferably used and dissolved in the medium to a final concentration of in the wells of 250 µg per ml.

15 The test can be performed in any suitable vessel e.g. in tubes or preferably in the wells of microtiter plates.

20 For triggering cytokine production in vitro the cell concentration is of critical importance. If the test is performed only at one cell concentration, then only some of the cytokines of interest will be optimally detected. If a series of cell concentration is used the complete picture of the cytokine pattern predicted above in " Summary of the
25 invention" is revealed.

30 For substances to be tested for effects in the system different concentrations are added to the blood. The highest concentration of the substance being non toxic to the cells indicated by inspection in microscope in presence of vital stain e.g. trypan blue is used as start concentration. Serial dilution of the substance is then performed down to a dilution of at least 1:1 000 and preferably down to 1:10 000, 1:100 000 or 1:1 000 000.

35 In cases where the substance is diluted in RPMI 1640 the appropriate control is medium only. When other diluents are

used, controls of the appropriate diluent at corresponding concentrations are used.

The vessels are incubated at 25-40°C, preferably at 37°C at 50-95%, preferably 90% humidity and preferably in the presence of with 2-15% CO₂, preferably 5%. An appropriate number of tubes or microtiter plates are prepared and are taken out of the incubator for testing at the intervals. Standard time intervals are 1 hour, up to 10 days; preferably 1 hour 1 day, 2 days and 4-6 days.

Proliferation assay can be made as follows. One set of plates is used to estimate the proliferative activity of the donor cells spontaneously (=medium) or substance dissolvent controls), in response to mitogens and in response to samples of test substances at various concentrations This can be done as follows or by any other suitable method. At time intervals plates are tested, and to each well in the plate is added labeled nucleic acid precursor such as ³H-thymidine dissolved in the medium. The plates are incubated for additional 4 hours at 37°C for incorporation of labeled nucleic acid precursor. The cells are then collected using a Skatron cell harvester (Skatron, Lier, Norway) and Titertek paper filters or any other suitable method. The result of this procedure is that the DNA of the cells is captured in the filter paper, whereas the ³H-thymidin not incorporated in the DNA passes through the filter and goes with the waste washing water. The radioactivity in the filter papers is thus proportional to the DNA synthesis that occurred during the 4 hours preceding the harvesting procedure. The radioactivity of the filter papers is determined in a Packard liquid scintillation counter, and thymidine incorporation is expressed as counts per minute (cpm) The radioactivity or any other label of the precursor can be measured in DNA harvested by any other mechanical or chemical way and measured by any suitable isotopic or chemical instrument.

Cytokine assays: Another set of plates is prepared with the same design as in "Culture protocol" above. Also here, plates are removed from the incubator at various time intervals, and cytokines released from the cultured cells are measured in the supernatants obtained after centrifugation of the plates at 650 g for 10 minutes. The supernatants can either be tested immediately or stored at -20°C until tested. For cytokine determinations diagnostic kits of various origins are used. Patent is claimed for this procedure using any available or newly constructed test using cytokine quantitation including bioassays, immunoassays or chemical assays or other.

The instructions of the manufacturers are followed. All these tests are enzyme immunoassays (EIA:s). The principle is that microtiter plate wells are labeled with a cytokine specific capture antibody. If cytokine is present in the samples added to the wells it will be captured to the bottom of the well. To quantitate how much cytokine that has been captured a second antibody, labeled with an enzyme is added to the wells. The reaction is thereafter measured as colour developed after addition of a substrate for the enzyme. The values for the test wells is compared with a standard curves obtained from a series of known amounts of cytokine. The values for control wells with no substance added to the cells are used as background levels for the tests. Positive indications are recorded when values above 2 SD of the variation are obtained.

The invention also concerns a kit comprising one or more reagents recognizing the alarm cytokines of class zero, and/or the cytokines of class I type, and/or the cytokines of class IV. Examples of such cytokines are mentioned in the last two paragraphs on page 4 and the first paragraph on page 5. Reagents reacting for the presence of neopterin and IL-8 are preferred.

These reagents may be antibodies or any other reagents that are sensitive for these substances. The reagents may be supported by a carrier such as a strip, titer plates, micro-titer plates, Eliza plates, test tubes etc. The carriers may be of different sizes. The carrier material may be any solid or semi-solid material that does not interfere with the reaction between the reagent and the cytokine. The carrier can take on a variety of shapes and compositions, including microparticles, beads, porous and impermeable strips and membranes, the interior surface of reaction vessels such as test tubes and microtiter plates, and the like. Microtiter plates and beads may be of plastic, such as styrene or acryl-polymers or glass. Nitrocellulose can be used, preferably in the form of filters, strips or discs. Means for attaching a desired reaction partner to a selected solid support will be a matter of routine skill to the worker in the field. It is also possible to use flow cytometer.

The invention will now be better described with the following non limiting examples.

Example

Blood donors and blood collection: Five healthy blood donors from both sexes ranging in age between 25 and 55 years are used. Blood is collected by venous puncture into endotoxin free heparin tubes (Coatech, Ljungby, Sweden) that contains 120 IU heparin and are filled with 4 ml of blood. None of the blood donors are allowed to have a history of allergy or atopic excema or any indication of ongoing disease of any kind.

Cell analysis: Total and differential white blood cell counts are measured with Coulter STKS equipment. The five donors had total white blood cell counts of 9,8; 7,1; 9,8; 6,9; 5,8 · 10⁹/l.

Culture protocoll: The whole blood is diluted serially 1:2, 1:3, 1:20, 1:4, and 1:10 in tissue culture medium, RPMI 1640

supplemented with 2 mM glutamine, 50U/ml of penicillin and 50Ug/ml of streptomycin. 100 ul of blood is added to the wells of Nunclon microtiter plates. The mitogen phytohemagglutinin (PHA-L) (L-4144, Sigma St Louis USA) is dissolved in RPMI 1640 and 50 ul of the solution is added to the wells so that a final amount of 25 µg per well is achieved.

For substances to be tested for effects in the system different concentration are added to the blood. The highest concentration of the substance being non toxic to the cells indicated by inspection in microscope in presence of vital stain e.g. trypan blue is used as start concentration. The following substances and concentrations were used:

O = Triton x 100 in concentrations 1 : 2000, 1 : 4000,

1 : 8000, 1 : 16000, 1 : 32000, 1 : 64000, 1 : 128000

IV = NiCl₂ 100 µg, 10 µg, 1 µg, 0,1 µg, 0,01 and 0,001 µg per ml

I = mite extract (1 : 10, 1 : 20, 1 : 40, 1 : 80

Pos = PHA 25 µg per well (200 µl)

In cases where the substance is diluted in RPMI 1640 the appropriate control is medium only. When other diluents are used, controls of the appropriate diluent at corresponding concentrations are used.

Incubation: The microtiter plates are incubated at 37°C at 90% humidity and with 5% CO₂. An appropriate number of identical plates are prepared and are taken out of the incubator for testing at intervals of, 1 day, 2 days and 6 days.

Proliferation assay: One set of plates is used to estimate the proliferative activity of the donor cells spontaneously (=medium or substance dissolvent controls), in response to PHA, and in response to samples of test substances at various concentrations. The highest concentration of substance non toxic to the cells indicated by inspection in microscope in presence of vital stain (e.g. trypan blue) is used as start

concentration. Serial dilutions of the sub-stance is then performed as stated above.

To each well in the plate was added 1 uCi of ^3H -thymidine (Amersham International, Amersham UK; 50 Ci/ mM) in 50 ul of RPMI 1640. The plates were incubated for addional 4 hours at 37°C. The cells were then collected using a Skatron cell harvester (Skatron, Lier, Norway) and Titertek paper filters. The result of his procedure is that the DNA of the cells is stuck in the filter paper, wheras the ^3H -thymidin not incorporated in the DNA passes through the filter and goes with the waste washing water. The radioactivity in the filter papers is thus proportional to the DNA synthesis that occured during the 4 hours preceding the harvesting procedure. The radiactivity of the filter papers is determined in a Packard liquid scintillation counter, and thymidine incorporation was expressed as counts per minute (cpm).

Cytokine assays: Another set of plates is prepared with the same design as in "Culture protocoll " above. Cytokines released from the cultured cells are measured in the supernatants obtained after centrifugation of the plates at 650 g for 10 minutes. The supernatants were either tested immediately or stored at -20°C until tested.

For information only is here mentioned that in the tests performed the following commercial kits and manufacures were used.

IL-1beta: Immunotech, Chromgenix

IL-2: Immunotech, Chromgenix

IL-4: R&D Systems

Neopterin: Henning Berlin

IL-6: Immunotech, Chromgenix

IL-8: Assay Res. Inc

Interferon-gamma: Genzyme

sCD8: T Cell Diagnostics

sIL-2R Immunotech

TNF: Medgenix

IL-10: Medgenix

The instructions of the manufacturers were followed. All these tests are enzyme immunoassays (EIA:s). The principle is that microtiter plate wells are labeled with a cytokine specific capture antibody. If cytokine is present in the samples added to the wells it will be captured to the bottom of the well. To quantitate how much cytokine that has been captured a second antibody, labeled with an enzyme is added to the wells. The reaction is thereafter measured as colour developed after addition of a substrate for the enzyme. The values for the test wells is compared with a standard curve obtained by adding a series of known amounts of cytokine. The values obtained for control wells with no substance added are used as background values for cytokine levels and proliferation as measured by ³H-thymidin incorporation. Positive indications are recorded when values above 2 SD of the variation of the controls are obtained.

The results from testing three substances with previously from clinical experience known properties, but not previously tested with the method are shown:

- 0. Triton X-100 This is a detergent with tissue damaging effects. Dissolves cell membranes.
- I. Dust mite antigen (Soluprick, ALK Sverige AB). This preparation is made from an extract of dust mites and is used clinically to test for immediate type allergy by intracutaneous injection.
- IV. Nickel chloride (Sigma St Louis, USA). This metal is wellknown as a cause of delayed type allergy (contact excema) in humans and experimental animals.
- Plus. The positive indicator of responsiveness of the system the calibrator was the mitogen PHA (Sigma, St louis, USA).

In vitro performance test - Table I

Values for 11 cytokines and ³H-Thymidine Proliferation. Obtained after stimulation of blood from 5 normal humans. All values are related to medium control given the value 100.

	IL-1	TNF	IL-6	IL-8	IL-2	STL-2R	SCD8	IL-10	IL-4	IFN γ	Neop- terin	Prolif. Index
O	630	400	460	200	85	115	95	95	85	120	110	200
IV	1165	225	660	380	445	130	110	800	80	1220	90	1340
I	320	240	250	100	900	120	85	640	900	800	560	120
Plus	690	420	1620	780	750	1820	940	1250	820	800	440	2600

The highest value as compared to medium control obtained at any time or concentration for each substance is used as indicator value in the table and related to medium control put to value 100 index.

5

Results: In summary the results of a performance example are presented below and the stimulatory effects were quite different for the different compounds.

10 0. Triton X-100 induced proliferation. Cytokines of the unspecific tissue toxic group were induced i.e. IL-1, IL-6 and TNF. This is a positive tissue irritant.

I. Dust mite: Induced no proliferative response.

15 Substances secreted into supernatants were the unspecific inflammatory IL-1, TNF and IL-6 and of immune lymphocyte class the IL-2, IL-4, IL-10 and IFN-gamma. Furthermore the Neopterin typical for induction of specific immune responses of a late mature stage. There is a potential for antibody and
20 IgE production.

IV. Nickel chloride: Induced a low proliferative response. The cytokines induced were the unspecific inflammatory mediator IL-1, IL-6, TNF and IL-8. Of the immune lymphocyte
25 class IFN-gamma, IL-2 and IL-10 were induced, but notably no Neopterin.

Plus. PHA: Induced a strong proliferative response indicated by ³H-thymidine incorporation and also cell types of cytokines were induced. The substances secreted in the supernatants included: Cytokines of primary inflammatory type as
30 IL-1, IL-6, TNF and IL-8. Cytokines of the types typical for specific immune responses both early and late as neopterin, IFN-gamma IL-2 and IL-4 and IL-10 typical of both early and late responses were induced.

Claims

1. A process for in vitro evaluation of a potentially
allergenic or tissue irritating substances, characterized in
5 that blood cells are cultivated in the presence of serial
dilutions of the substance whereby the highest concentration
of the substance being non toxic to the cells is serial
diluted, cell proliferation is measured and the presence of
cytokines is measured whereby :

10

the presence of one or more of the alarm cytokines of class 0
only is an indication of tissue damage and chemical toxic
effects,

15

the presence of one or more alarm cytokines of class 0 and
possibly one or more cytokines of class IV type but not
neopterin is an indication of delayed type hypersensitivity
such as cellular immunity, delayed allergy and contact eczema
and

20

the presence of one or more alarm cytokines of class 0 and at
least neopterin and possibly one or more cytokines of class I
is an indication of immediate type hypersensitivity such as
asthma, hay fever, urticaria and rhinitis.

25

2. A process according to claims 1, characterized in that the
cytokines of the alarm groupe is selected from cytokines
indicating damage to connective tissue, fibroblasts, endo-
thelial cells, epithelial cells and unspecific iflammatory
30 white blood cells such as IL 1, IL-12, IL 6, TNF;

35

the cytokines of class IV type is selected from cytokines
connected with cell mediated T-cells immunity such as IL-2,
IL8, IL-10. IFN-gamma, IL-4, IL-5, and souluble products such
as sCD8 and sIL-2R, but not neopterin;

the cytokines of class I type are selected among cytokines of
an immune response type from T and B-lymphocytes and in-

inflammatory cells such as IFN-gamma, IL-2, IL-10 , IL-4, IL-5 and soluble products such as sCD8 and sIL-2R and neopterin.

3. A process according to claim 1 and/or 2, characterized in
5 that the substance to be tested is presented in the highest concentration being non toxic and in a serial dilution thereof at least 1:1000.

4. A process according to claim 3, characterized in that the
10 serial dilution is performed down to a dilution of at least 1:10 000 to 1: 1 000 000.

5. A process according to claim 1 and/or 2, characterized in
15 that the blood is diluted serially from 1:1 to 1:1000, preferably from 1:2 to 1:100.

6. A process according to claim 1 characterized in that
whole blood is treated to not coagulate, diluted 1:2-1:100 in
tissue culture medium preferably supplemented with glutamine
20 and/or an antibioticum, a marked nucleic acid precursor is added,

the substance to be tested is serially diluted down to a
dilution of 1: 1000 to 1:1 000 000 from the lowest con-
25 centration of the substance being non toxic to the cells preferably as indicted by inspection in microscope in presence of vital stain and added in different concentrations and incubated at 25C-40C, and tested at time intervals from 1 hour to 6 days by

30 certifying that proliferation has taken place preferably by collecting the cells on filter paper or harvesting nucleic acid by any other mechanical or chemical method and analysing the labeled nucleic acid precursor and

35 analysing cytokines by estimating the presence of

one or more of the alarm cytokines of class 0 only is an indication of tissue damage and chemical toxic effects,

5 one or more alarm cytokines of class 0 and one or more cytokines of class IV type but not neopterin is an indication of delayed type hypersensitivity such as cellular immunity, delayed allergy and contact eczema and

10 one or more alarm cytokines of class 0 and at least neopterin and possibly one or more cytokines of class I is an indication of immediate type hypersensitivity such as asthma, hay fever and urticaria.

15 7. A process for in vitro evaluation of an potentially allergenic or tissue irritating substance characterized in that blood cells are cultivated in the presence of serial dilutions of the substance and in the presence of neopterin is analysed in a manner known per se whereby the presence of neopterin is an indication of immediate type hypersensitivity
20 such as asthma, hay fever and urticaria.

8. Use of IL-8 and/or neopterin for analysing the presence of the inflammatory immune reaction type IV and/or the allergic immune reaction type I.
25

9. A reagent kit for use in the process according to anyone of claims 1-7, characterized in that it comprises one or more reagents recognizing cytokines.

30 10. A reagent kit according to claim 9, characterized in that it comprises a reagent such as an antibody, recognizing IL-8 and/or neopterin.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/00902

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/68, G01N 33/50, C12Q 1/02, C12N 5/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, WPIL, CA, SCISEARCH, US PATENTS FULLTEXT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Immunology, Volume 85, 1995, M. Imada et al, "Allergen-stimulated interleukin-4 and interferon- γ production in primary culture: responses of subjects with allergic rhinitis and normal controls" page 373 - page 380 --	1-7
A	In Vitro Toxicology, Volume 5, No 4, 1992, K. Müller-Decker et al, "Development of an In Vitro Alternative Assay to the Draize Skin Irritancy Test Using Human Keratinocyte-derived Proinflammatory Key Mediators and Cell Viability as Test Parameters" page 191 --	1-7

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- * "A" document defining the general state of the art which is not considered to be of particular relevance
- * "B" earlier document but published on or after the international filing date
- * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * "O" document referring to an oral disclosure, use, exhibition or other means
- * "P" document published prior to the international filing date but later than the priority date claimed

* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

* "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

* "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

* "&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

3 February 1997

10 -02- 1997

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/00902

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Immunology Letters, Volume 46, 1995, Robert V. House et al, "In vitro evaluation of fentanyl and meperidine for immunomodulatory activity" --	1-7
X	US 5439799 A (WILFRIED RAUTENBERG ET AL), 8 August 1995 (08.08.95), see claims 14-15 --	9-10
X	Dialog Information Services, file 155, Medline, Dialog accession no. 09433182, Medline accession no. 95363182, Larsen CG et al: "The delayed-type hypersensitivity reaction is dependent on IL-8. Inhibition of a tuberculin skin reaction by an anti-IL-8 monoclonal antibody", J Immunol (UNITED STATES) Aug 15 1995, 155 (4) p2151-7 --	8
A	Dialog Information Services, file 155, Medline, Dialog accession no. 06865075, Medline accession no. 89167075, Dewitte JD et al: "Improved HPLC determination of urinary neopterin", Biomed Chromatogr (England) 1987, 2 (5) p183-8 --	1-8
A	Dialog Information Services, file 155, Medline, Dialog accession no. 06093302, Medline accession no. 87067302, Dewitte JD et al "Assay of urinary neopterin in hypersensitivity pneumopathies (letter)", Presse Med (FRANCE) Nov 1 1986, 15 (38) p1932 -- -----	1-8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/00902

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

As is stated in Annex B to Administrative Instruction under the PCT, in force July 1, 1992 (PCT GAZETTE 1992, June 25, pages 7062-9, see page 7063 and example 5) unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features"-i.e. features that define a contribution which each of the inventions makes over the prior art.

A search for this "special technical feature" mentioned in PCT Rule 13.2 among the independent claims did not reveal such a unifying, novel technical feature, Accordingly, the following inventions were found:

Invention A, claims 1-7, 8 (partially), 9, 10 (partially) pertains to a process for in vitro evaluation of potentially allergenic or tissue irritating substances, whereby blood cells are cultivated in the presence of serial dilutions of the substance and the presence of cytokines i.a. neopterin, is measured.

Invention B, claim 8, (partially) and 10 (partially) concerns the use of IL-8 as an analytic substance for analysing the presence of the inflammatory immune reaction type IV.

Since the measurement of IL-8 is not considered to be a special technical feature of the process according to invention A there seem to be no unifying technical feature between the inventions.

However, the international search report covers both inventions.

28/10/96

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Form PCT/ISA/210 (patent family annex) (July 1992)